A putative gene cluster for aminoarabinose biosynthesis is essential for Burkholderia cenocepacia viability

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Cationic antimicrobial peptides (APs), a group of structurally diverse molecules found in all eukaryotes (17, 34), can kill gram-positive and gram-negative bacteria, fungi, and viruses and also modulate innate immune responses (30). In gram-negative bacteria, APs interact with lipopolysaccharide (LPS) and via the self-promoted uptake pathway reach targets in the plasma membrane (16). LPS is a major surface component of gram-negative bacteria, consisting of O antigen polysaccharide, core oligosaccharide, and lipid A (29). Phosphate groups covalently attached to residues on the lipid A and core oligosaccharide provide sites for electrostatic interactions with APs (18). Bacteria can resist APs by modifying their lipid A with 4-amino-4-deoxy-l-arabinose (Ara4N), effectively reducing the net negative charge of LPS molecules. In all species examined to date, this is a highly regulated process in response to environmental Mg²⁺ limitation, excess Fe⁴⁺, or the presence of APs (1, 8, 9, 12, 15, 24). However, LPS modification with Ara4N is dispensable for growth in vitro under routine laboratory conditions.

Burkholderia cenocepacia belongs to the B. cepacia complex, a group of closely related environmental bacteria that are emerging opportunistic pathogens of cystic fibrosis patients and other immunocompromised individuals (23). They are highly resistant to the majority of clinically useful antimicrobials and also resistant to APs even at concentrations that kill other bacteria with Ara4N-modified LPS (21). Very little is known about the mechanism of resistance to APs in Burkholderia spp. LPS from various Burkholderia species, such as B. cepacia, B. caryophylli, and B. cenocepacia, contains Ara4N residues in the lipid A and also the unusual glycerol-talo-olurosanic acid (Ko) residue in the inner-core oligosaccharide (10, 13, 19, 20, 25, 26, 31, 32). Here, we show that a putative gene cluster for Ara4N biosynthesis and LPS modification is essential for the viability of B. cenocepacia.

Using a conditional mutagenesis strategy we demonstrate here that a gene cluster encoding putative aminoarabinose (Ara4N) biosynthesis enzymes is essential for the viability of Burkholderia cenocepacia. Loss of viability is associated with dramatic changes in bacterial cell morphology and ultrastructure, increased permeability to propidium iodide, and sensitivity to sodium dodecyl sulfate, suggesting a general cell envelope defect caused by the lack of Ara4N.
nomic manipulations. RT-PCR analysis also revealed that the \(\text{arn}\) cluster in both strains was expressed regardless of polymyxin B challenge (data not shown), indicating that it is not subjected to the same transcriptional regulation as has been observed in all other bacteria examined to date (1, 24). To facilitate genetic manipulations the remaining studies were performed using strain K56-2, which is also highly resistant to APs (21).

The putative Ara4N biosynthesis cluster is essential for viability of \(B.\ cenocepacia\). Repeated attempts to mutagenize \(\text{arnT, arnB, arnC, and BCAL1935}\) by insertional inactivation using the pGP\(\text{H9024}\) suicide plasmid (11) containing internal fragments from each target gene consistently failed. In contrast, the genes BCAL1928 and BCAL1936 that flank each end of the putative \(\text{arn}\) cluster were readily mutated. We hypothesized that the putative \(\text{arn}\) genes perform one or more functions that are essential for the viability of \(B.\ cenocepacia\). To confirm the essentiality of the \(\text{arn}\) genes, we constructed plasmid pSC200, which enables the delivery of the rhamnose-inducible \(P_{\text{rhaB}}\) promoter into the chromosome to drive the expression of a targeted gene. The construction of pSC200 was done by combining the multiple-cloning site, ori\(_{\text{R6K}}\), and \(\text{mob}\) genes from pGP\(\text{H9024}\) (11) with the \(P_{\text{rhaB}}\) rhamnose-inducible promoter, \(\text{rhaR, rhaS,}\) and the \(\text{dhfr}\) cassette from pSCrhaB2 (7). Details on the construction of this plasmid are available from the authors upon request. Several 300-bp fragments spanning the 5' region of each targeted gene were cloned into pSC200. The resulting plasmids were transferred into \(B.\ cenocepacia\) K56-2 by triparental mating, and the exconjugants were plated on LB agar plates supplemented with trimethoprim (Tp) (100 \(\mu\)g/ml), gentamicin (50 \(\mu\)g/ml), and 0.5% (wt/vol) rhamnose. The correct insertion in the \(B.\ cenocepacia\) chromosome was confirmed by colony PCR and Southern blot hybridization. This strategy created conditional mutants in which the expression of the targeted gene depended on the rhamnose concentration in the medium. We have shown that the \(P_{\text{rhaB}}\) promoter is useful for characterizing essential operons in \(B.\ cenocepacia\) (6). Thus, we constructed conditional mutants by insertion of pSC200 derivatives upstream of \(\text{arnT}\) (strain XOA11) and \(\text{arnB}\) (strain XOA12) as well as a mutant in which this plasmid interrupts the gene locus BCAL1928 (strain XOA10) and which served as a negative control (Fig. 1A). Also, as a positive control for essentiality, a pSC200 derivative was used to target the conserved essential gene \(\text{dxs}\) encoding a key enzyme for the synthesis of undecaprenyl phosphate (2, 28), resulting in strain STC280.

The conditional mutants were grown at 37°C in M9 minimal medium (42 mM Na\(_2\)HPO\(_4\), 22 mM KH\(_2\)PO\(_4\), 8 mM NaCl, 10 mM NH\(_4\)Cl) supplemented with (final concentrations) yeast extract (5 mg/ml), Casamino Acids (2 mg/ml), vitamin B\(_1\) (2 \(\mu\)g/ml), tryptophan (20 \(\mu\)g/ml), CaCl\(_2\) (1 \(\mu\)M), and 0.5% (wt/vol) glycerol plus 0.5% (wt/vol) glucose or 0.5% (wt/vol) rhamnose and Tp (100 \(\mu\)g/ml) when required. An aliquot of an overnight culture in M9 medium with rhamnose was spun down and washed three times with sterile phosphate-buffered saline (PBS), resuspended in PBS, and adjusted to an optical density at 600 nm (OD\(_{600}\)) of 1. Drops (10 \(\mu\)l) of undiluted solution and 10\(^{-1}\), 10\(^{-2}\), 10\(^{-3}\), and 10\(^{-4}\) dilutions were plated onto M9 agar plates supplemented with 0.5% (wt/vol) glucose or 0.5% (wt/vol) rhamnose and incubated at 37°C. Strain STC280 grew in the presence of rhamnose (permissive condition) but failed to grow in the presence of glucose (nonpermissive condition), as expected for a mutant with an essential gene under the control of \(P_{\text{rhaR}}\). A similar growth phenotype was found for XOA11 and XOA12, but XOA10 grew equally well in the presence of rhamnose or glucose (Fig. 2A). We
precursor for core oligosaccharide biosynthesis (21). To as-
glycero-manno-
part of the biosynthesis of the ADP-
ugd
dium with glucose (Fig. 2A), indicating that
mutant did not show a conditional lethal phenotype in me-
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additional
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J2315 genome revealed the presence of two
not essential for bacterial viability. A BLAST analysis of the
ugd
glucose dehydrogenase activities.

and BCAM2034), which we assume provide redundant UDP-
the two transcriptional units of the putative
arn
therefore concluded that expression of one or more genes in
the two transcriptional units of the putative arn cluster is
inispensable in B. cenocepacia K56-2.

The first biochemical reaction for the biosynthesis of
Ara4N is the conversion of UDP-glucose into UDP-gluc-
uronic acid by the enzyme UDP-glucose 6-dehydrogenase
(Ugd or PmrE). In previous work, we characterized a gene
operon containing a ugd homologue (BCAL2946) and genes
coding modification of glycerol-manno-heptose, which are
part of the biosynthesis of the ADP-glycerol-manno-heptose
precursor for core oligosaccharide biosynthesis (21). To
assess whether or not ugd_BCAL2946 is also essential, we con-
structed a conditional ugd_BCAL2946 mutant (SAL10). This
mutant did not show a conditional lethal phenotype in me-
dium with glucose (Fig. 2A), indicating that ugd_BCAL2946 is
not essential for bacterial viability. A BLAST analysis of the
B. cenocepacia J2315 genome revealed the presence of two
additional ugd homologues in chromosome 2 (BCAM0855
and BCAM2034), which we assume provide redundant UDP-
glucose dehydrogenase activities.

Loss of viability is associated with morphological changes
in the bacterial cells and defects in cell envelope permeability.
The essentiality of Ara4N synthesis was further demonstrated
by depletion experiments performed using liquid medium (Fig.
2B). Eight-hour cultures were diluted to an OD600 of 0.001 in
M9 medium supplemented with 5% (wt/vol) yeast extract and
0.5% (wt/vol) rhamnose and incubated at 37°C for 11 h. Cells
from these cultures were spun down, washed three times with
sterile PBS, and diluted with fresh M9 medium supplemented
with 5% (wt/vol) yeast extract and 0.5% (wt/vol) glucose at a
final OD600 of 0.13. Growing cells were subcultured with fresh
medium containing 0.5% (wt/vol) glucose at 4 h and monitored
for growth for another 6 h. Growth curves of the wild-type
strain and all the mutants in permissive conditions were ident-
ical (data not shown). After serial passages in medium with
glucose, the wild type and the XOA10 mutant also grew to
similar levels (Fig. 2B). In contrast, after serial passage in
medium with glucose of XOA11 and XOA12 cultures, the
turbidity of the cultures rapidly reached a plateau compared to
wild-type and XOA10 control strain results. The results indi-
cate that XOA11 and XOA12 had stopped growing after 6 h
(roughly corresponding to six generations). Phase-contrast mi-
croscopy and fluorescent staining with Syto9 and propidium
iodide (LIVE/DEAD BacLight bacterial viability kit; Molecular
Probes, Invitrogen Detection Technologies, Eugene, OR)
were carried out to investigate the bacterial cell morphology
and to assess whether or not the cell envelope was compro-
mised. After 8 h in nonpermissive conditions, strains XOA11
and XOA12, but not K56-2 and XOA10, formed chains of cells
and the cultures contained flocculent particulate material in-
dicative of cell lysis. Also, 50% of XOA11 and 58% of XOA12
bacterial cells were permeable to propidium iodide in contrast
to 5% to 10% of the control strains, suggesting a loss of
viability and a compromised cell envelope in the arn condi-
tional mutants (Fig. 3). No differences in morphology or via-
bility were observed between these same strains grown over-
night under permissive conditions before being transferred to
the nonpermissive medium for the depletion experiments (data
not shown).

Ultrastructural analysis of bacterial cells after 8 h following
depletion revealed accumulations of membranous material in
strains XOA11 and XOA12 (Fig. 4), suggesting that Ara4N is
required for the proper assembly of the outer membrane. Fur-
thermore, empty cells and cells with cell division defects could
be observed in the mutant cultures (Fig. 4). To obtain further
evidence that the envelope was compromised in XOA11 and
XOA12 under nonpermissive conditions, bacteria were grown
in the presence of 0.05% (wt/vol) sodium dodecyl sulfate
(SDS). For these experiments, cells were diluted to an OD600
of 0.1 and dilutions were plated for colony counts on M9 agar
plates supplemented with 5% (wt/vol) yeast extract, 0.5% (wt/
vol) rhamnose, and Tp (100 μg/ml) with or without 0.05%
(wt/vol) SDS. The survival rate of K56-2 cells in the presence
of SDS was 1.2% of the initial inoculum, while the survival rates
of XOA11 and XOA12 cells were 0.09% and 0.08%, respec-
tively. Together, these experiments demonstrate that the
putative arn gene cluster is essential for B. cenocepacia viability
and that its function is required for an intact cell envelope. It
is possible that LPS modifications with Ara4N are an essential
requirement for the stability of the outer membrane in this

FIG. 2. (A) Conditional lethal phenotype of strains XOA11 and
XOA12 on M9 agar plates supplemented with 0.5% (wt/vol) rhamnose
or 0.5% (wt/vol) glucose. XOA11 and XOA12 grow only in the
presence of rhamnose, while XOA10 and SAL10 grow equally well in
either media. STC280 is shown as a control mutant for a known
essential gene. (B) Depletion experiments using strains K56-
2(pSCrhaB2), XOA10, XOA11, and XOA12 in nonpermissive
conditions. Growth was monitored every hour using a Klett-Summerson
colorimeter. The figure is representative of two independent experi-
ments with similar results.
FIG. 3. Microscopy and live-dead staining of strains K56-2, XOA10, XOA11, and XOA12 at 8 h of growth during the depletion experiments. Live bacteria appear fluorescent green, while dead bacteria and bacteria with compromised membranes appear fluorescent red. These experiments were carried out twice with similar results.
group of bacteria, which may explain at least in part their extreme resistance to APs. Recent structural studies of the lipid A moiety of B. cepacia (formerly genomovar I) and B. mallei revealed that only a proportion of lipid A species are nonstoichiometrically replaced with Ara4N (5, 31), although we do not know whether this is also the case for the lipid A from B. cenocepacia. Perhaps not all the lipid A molecules are required to be replaced with Ara4N to maintain the stability of the outer membrane. Furthermore, our results did not conclusively establish whether or not Ara4N is essential in the lipid A and/or the inner core oligosaccharide. The morphological defects of the mutants under nonpermissive conditions are strikingly similar to those found in Escherichia coli mutants with defects in the Imp/RlpB complex, which is essential for proper assembly of LPS in the outer membrane (33). Therefore, it is possible that the products encoded by the putative arm gene cluster in B. cenocepacia are also required for the assembly of LPS in this bacterium. A detailed biochemical analysis of the lipid A biosynthesis in B. cenocepacia, currently under way in our laboratories, will help to elucidate the role of these genes and will also confirm their predicted function in the synthesis of Ara4N.

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